Chapter

Streamlining the Fermentation Process Using Mixed Cultures

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Abstract

Fermentation technology is still being developed in all aspects, with the aim of improving the yields and qualities of products and reducing the costs of production. Increasing the yields of fermentation products can be accomplished by optimizing the factors that influence the process, including both the microbe itself and the environment. For example, the acetic acid production process from raw materials can be performed simultaneously with submerged batch fermentation using mixed cultures of anaerobic and facultative anaerobic *S. cerevisiae* and obligate aerobic *A. aceti*. This system is very simple because it only has one stage. In this system, efforts can be made to enhance the yields of acetic acid production, including evaluating the availability of nutrients in the medium and determining the optimum proportion of microbial abundance and agitation speed. Under optimal conditions, the resulting increases in acetic acid yields occur with high conversion efficiency. These results can then be applied on an industrial scale by integrating these findings with advanced technologies in the operating system.

Keywords: acetic acid production, *Acetobacter aceti*, aerobic submerged fermentation, mixed culture, *Saccharomyces cerevisiae*

1. Introduction

The fermentation industry has developed rapidly, especially as bioreactors have become the center of the process, as previously described [1]. The factors that have been the focus of development include the feeding of the bioreactor (batch, fed-batch, and continuous mode of operation), the use of microbial cultures (single strain or mixed culture processes), the availability of oxygen (aerobic, microaerobic, and anaerobic processes), and the mixing of the bioreactor during the process, particularly in the production of acetic acid. Acetic acid is produced from alcohol, and alcohol is produced from sugar. These two processes require different types of microorganisms. The microorganisms most commonly used in the fermentation of alcohol are yeasts, such as *Saccharomyces cerevisiae*, and bacteria, such as *Zymomonas mobilis*. However, for industrial fermentation, *Z. mobilis* appears to be inferior to *S. cerevisiae*, due to the reduced biomass production of the bacterium when pH decreases [2]. Commonly used acetic acid bacteria (AAB) include *Acetobacter* and *Gluconacetobacter*, two AAB genera that oxidize ethanol more easily than sugars [3], and exhibit resistance to high acetic acid concentrations and low pH [4]. For large-scale industries, the efficiency of the fermentation process design and operation continues to be developed, with the aim of improving the yields and qualities of the products and reducing the costs of production.

2. The development of fermentation technology in the production of acetic acid

In principle, the production of acetic acid from raw material is performed in two phases: the acetic acid fermentation process occurs under aerobic conditions, while alcoholic fermentation occurs under anaerobic conditions. Traditionally, the two processes are performed separately, under static and uncontrolled conditions [5, 6]. However, in its development, the production of acetic acid tends to occur in two or more stages, using either batch, fed-batch, or continuous types of operations. Many modifications have been made to the process, some of which are listed in **Table 1**. These modifications include the identification of alternative raw materials, the use

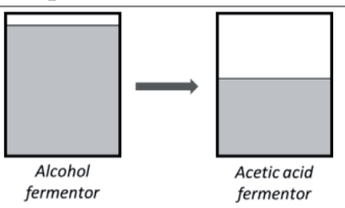
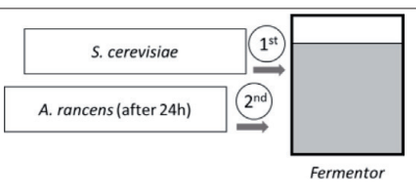
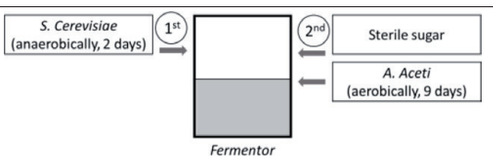
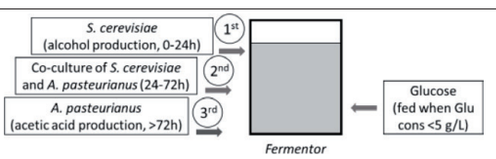
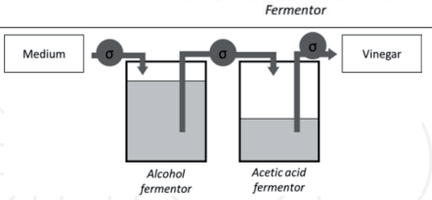
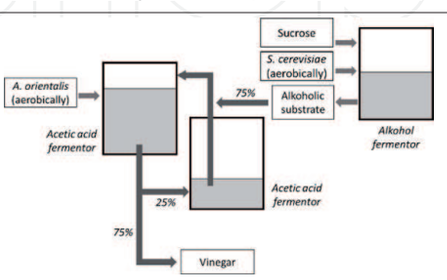
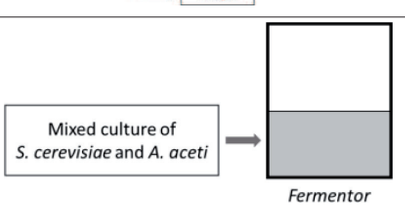
No.	Type of operation	Number of stages	Simplified schematic diagram of the fermentation setup	Ref.
1	Batch	2		[17]
2	Batch	2		[10]
3	Batch	2		[8]
4	Fed-batch (successive stage)	3		[16]
5	Continuous	1		[18]
6	Semicontinuous	2		[7]
7	Batch	1		[9]

Table 1.
The various fermentation processes used during acetic acid production from raw materials.

of different types of microorganisms, the implementation of different fermentation process operations and stages, and the manipulation of environmental conditions.

The identification of alternative raw materials is performed not only to identify new sources of material but also to address economic problems, such as the existence of surplus agricultural products, including onions [7], palm [8], or apples [9], and the utilization of sugar-containing waste materials, including pineapple peels [10]. The fermentation processes use different types of microorganisms. The identification and use of new types of microorganisms is performed with the aim of obtaining new strains with superior properties and abilities to produce high-quality and high-yield products [11–15]. Furthermore, various types of fermentation operations and modifications to the stages within these operations have been tested to determine the most simple and efficient methods capable of producing high yields because the operational procedures of acetic acid fermentation can be complicated and require a long time when multiple processes are required. Finally, the manipulation of environmental conditions, such as altering temperatures and aeration/agitation rates, is performed to obtain the optimal fermentation conditions [7, 9, 10, 16].

3. Enhanced acetic acid production from raw materials using mixed cultures during batch-type fermentation

Acetic acid fermentation has been studied using apples as a substrate. The fermentation was performed using submerged batch cultivation with mixed cultures of *S. cerevisiae* and *A. aceti*, which were inoculated simultaneously at the beginning of the process. These two microorganisms have different physiological properties: *S. cerevisiae* is a facultative anaerobe and requires anaerobic conditions to produce alcohol, and *A. aceti* is strictly aerobic. Because these microorganisms have opposing characteristics, using both cultures simultaneously is challenging, especially because acetic acid is a strong inhibitor of yeast, whereas yeast makes the medium anaerobic and unsuitable for AAB growth [5].

During this fermentation process, the first thing to be considered is the availability of sugar in the substrate, which represents a carbon source for the growth of the two microorganisms and the production of acetic acid. We must determine whether the sugar requirements are met by the substrate or whether sugar must be added, as described in previous studies [7, 8, 16]. Another factor that must be considered during this process is the inoculum ratio between the two microbes, as the strong competition between the two microbial groups must be anticipated and balanced to allow the production of acetic acid. Furthermore, because the different stages of acetic acid fermentation demand different oxygen requirements, the appropriate agitation speed is also important to consider.

3.1 Availability of sugar for microbial growth and acetic acid production

The microbial requirements for sugar during acetic acid fermentation and the availability of sugar in the substrate can be observed using different experiments, such as those shown in **Figure 1**. The system uses one-third of the working volume. Because this type of fermentation uses a batch culture with only one stage, all materials are added simultaneously at the beginning of the process, including sugar, at concentration of 0, 10, and 20% ($^{w}/_{v}$). Changes in the sugar, alcohol, and acetic acid contents and changes in the pH values during this process were evaluated, as previously described [9]. The results demonstrated that the conversions of sugar into alcohol and of alcohol into acetic acid were accompanied by decreases in the pH of the medium. This result indicates that the fermentation process has been successfully performed.

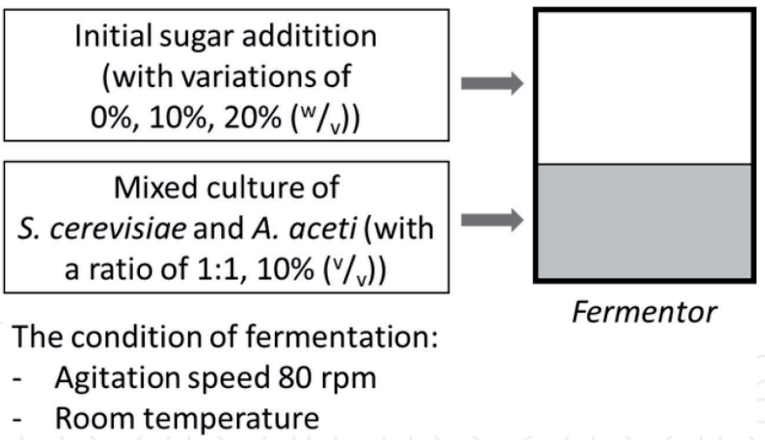


Figure 1.
Schematic diagram of the submerged batch fermentation process to evaluate the availability of sugar in the medium.

The percentage of initial sugar added to the medium ($^{w}/_{v}$)	0%	10%	20%
Conversion efficiency	233%	46.60%	6.40%

Table 2.
The conversion efficiency from sugar to acetic acid during 10 days of the fermentation process.

The sugar availability requirements during fermentation were determined by calculating the efficiency of the conversion of sugars into acetic acid (**Table 2**). According to **Table 2**, the conversion of sugar to acetic acid occurred with the highest efficiency when no sugar was added to the medium. Therefore, there is no need to add sugar to the medium because the substrate itself is sufficient to meet the needs of the microbes involved in the fermentation process and still produce a high acetic acid yield. The conversion efficiency of sugar into acetic acid during the fermentation process without the addition of sugar was greater than 100%. This result can be the result of the hydrolysis of starches contained in the medium, either chemically due to the decrease in pH [19, 20] or by *S. cerevisiae* to support growth and metabolic activity [21]. In addition to using glucose as its primary substrate, *S. cerevisiae* is able to grow on a wide range of carbon compounds, is able to metabolize some carbohydrates after they have undergone extracellular hydrolysis, and is able to ensure the efficient metabolism of those hydrolyzed carbohydrates [22]. Therefore, the addition of sugar to the fermentation medium is not required because *S. cerevisiae* is able to decompose and utilize the sugars that already exist in apples, which is evident from the relatively stable fermentative sugar content found in the fermentation medium during the fermentation process [9]. These results suggest that the nutrients contained in the apples were sufficient to support the maximum activity levels of the microbes.

In general, a higher sugar concentration in the medium results in the formation of a greater acetic acid content. However, excess sugar in the fermentation medium will not increase the microbial activity above its maximum threshold, and high sugar concentrations can limit the production of yeast biomass [23]. In addition, high levels of sugar can create anaerobic or microaerobic environmental conditions, which can inhibit the growth and activity of aerobic obligate bacteria, such as *A. aceti*, which is not optimal for acetic acid production. Thus, the availability of complex forms of sugar within the natural medium presents the advantage of providing a gradual carbon source to meet the needs of microbes.

3.2 Inoculum ratio of *S. cerevisiae* and *A. aceti*

In addition to the availability of sugar in the medium, the other factor that must be considered when performing acetic acid fermentations using mixed cultures is the optimal inoculum ratio of all cultures involved; in this case, *S. cerevisiae* and *A. aceti* were used. Because the two groups of microbes have different physiological properties, especially in terms of oxygen requirements, they also have different needs for carbon, different metabolic properties, and different growth rates. As mentioned above, *S. cerevisiae* is a facultative anaerobe that is able to grow on a wide range of carbon compounds and is able to produce alcohol under anaerobic conditions, whereas *A. aceti* is an obligate aerobe that is able to use ethanol, glycerol, and glucose as carbon sources for growth but is unable to hydrolyze lactose and starch and can oxidize ethanol to acetic acid and acetate to CO_2 and H_2O [4, 24]. Moreover, *S. cerevisiae* has a longer growth rate than *A. aceti* [9]. The metabolism and physiology of these two microbes have been described previously, in detail [4, 22, 24–26]. With these differences, the regulation of species dominance in mixed cultures by adjusting the inoculum ratios is expected to result in a syntrophic state that maximizes the production of acetic acid.

An example of an experimental design to determine the best ratio of the cultures used during acetic acid fermentation is shown in **Figure 2**. The ratios of *S. cerevisiae* and *A. aceti* cultures used were 3:7, 1:1, and 7:3. The performances of these microbes when used at different ratios during acetic acid production can be observed by measuring the changes in acetic acid contents and pH values during the process (**Figure 3**). The results showed that the highest acetic acid concentration with the lowest pH value was achieved on day 8 using mixed cultures of *S. cerevisiae* and *A. aceti* at a 7:3 ratio.

According to **Figure 3**, the acetic acid levels produced by the ratio of the 3:7 of *S. cerevisiae* to *A. aceti* are higher at the beginning of the process than those produced by the other ratios. In this period, the dominance of *A. aceti* over *S. cerevisiae* results in *A. aceti* rapidly utilizing glucose to convert the ethanol produced by *S. cerevisiae* into acetic acid. According to Maier [26], the initial inoculum size controls the length of the lag phase. However, during the next stage, the resulting acetic acid contents decreased. The larger ratio of *A. aceti* causes this microbe to require more nutrients, which the smaller ratio of *S. cerevisiae* cannot provide. The limited nutrients available to *A. aceti* result in suboptimal cell growth and enzymatic activity, causing the metabolic processes of *A. aceti* to not work properly and the resulting

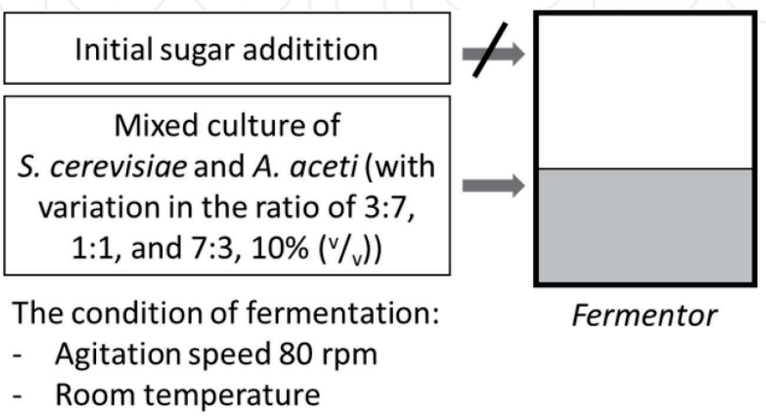


Figure 2.
Schematic diagram of the submerged batch fermentation process to determine the optimum inoculum ratio for the cultures used.

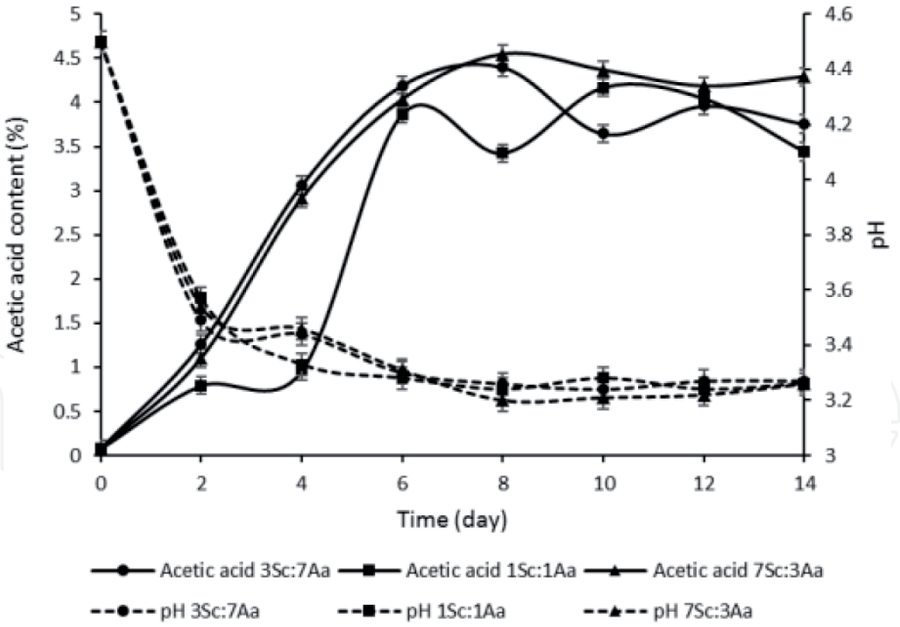


Figure 3.
Changes in the acetic acid contents and pH values with variations in the inoculum ratios between S. cerevisiae and A. aceti during the fermentation process.

acetic acid levels to decrease. Moreover, the acetic acid that is already produced undergoes overoxidation by *A. aceti* via the tricarboxylic acid (TCA) cycle [27].

The highest level of acetic acid was achieved on day 8, using the 7:3 inoculum ratio of *S. cerevisiae* to *A. aceti*. At the beginning of the fermentation process, the acetic acid concentration for this ratio was lower than for the 3:7 inoculum ratio, due to the dominance of *S. cerevisiae*. However, under aerobic conditions, *S. cerevisiae* is still able to produce alcohol in small amounts, and the large population of *S. cerevisiae* cells can produce enough alcohol to meet the nutrient requirements of *A. aceti*. During the later stages, the low levels of oxygen consumption by *S. cerevisiae* during alcohol production cause the availability of oxygen in the medium to become sufficient for *A. aceti* growth, and the resulting acetic acid contents increase.

3.3 Agitation speed for optimal mixing

As explained above, under aerobic conditions, the fermentation process using mixed cultures can work well, as indicated by the greater than 100% conversion efficiency from sugar to acetic acid. These results were achieved using an agitation speed of 80 rpm. Agitation plays an important role in fermentation processes, causing surface renewal; aiding in the dissolution of oxygen found at the top of the fermentor; improving the transfer of oxygen, heat, and mass through the system; and maintaining homogeneous physical and chemical conditions within the medium [28, 29]. Thus, the effect of agitation speed on the production of acetic acid in this system was evaluated by examining agitation speeds of 80 and 160 rpm (**Figure 4**). The percentage of acetic acid produced from both treatments can be seen in **Figure 5**.

The results showed that faster agitation speeds consistently resulted in higher acetic acid contents. The highest acetic acid level, 6.47%, was achieved on day 10 using an agitation speed of 160 rpm. Agitation is an important parameter for all aerobic processes [29]. The purpose of agitation during a submerged fermentation process is to homogeneously increase the availability and solubility of oxygen in the medium. Increased dissolved oxygen concentrations, generated by increased

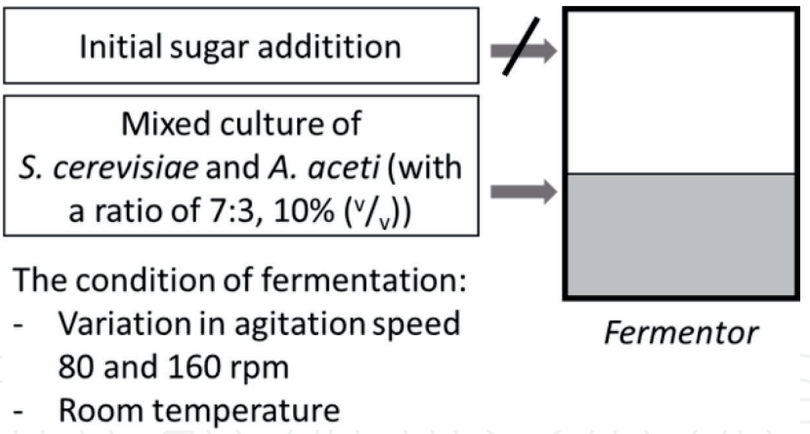


Figure 4.
Schematic diagram of the submerged batch fermentation process to determine the optimum agitation speed.

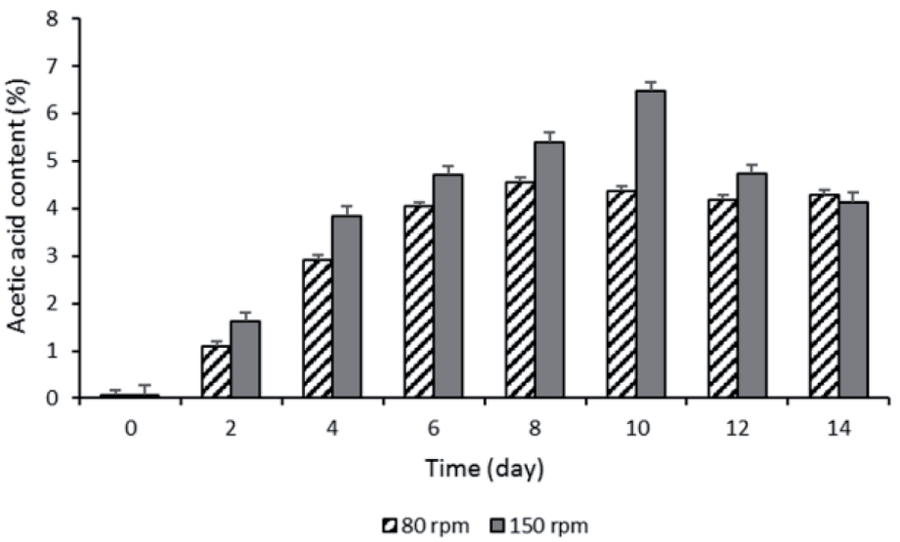


Figure 5.
The percentage of acetic acid produced from fermentation at different agitation speeds.

agitation speeds, resulted in a shortened lag time for cell growth and increased biomass formation [28]. Oxygen is needed not only by *A. aceti* but also by *S. cerevisiae* for growth [30, 31]. Massive oxygen consumption by both microbes simultaneously can create anaerobic conditions, causing *S. cerevisiae* to shift its metabolism from respiratory to fermentative and to produce alcohol. According to Navarro and Durand [32], during fermentation, yeast growth is rapidly stopped when the concentration of alcohol in the medium increases; however, fermentative activity is not entirely inhibited until high alcohol concentrations are reached. However, alcohol consumption by *A. aceti* prevents the concentration of alcohol in the medium from reaching the maximum value, preventing the inhibition of *S. cerevisiae* growth and activity, as indicated by the increase of glucose and alcohol contents in the medium. However, oxygen remains available in the medium, due to rapid agitation, allowing the growth and the activity of *A. aceti* to remain at high levels. *A. aceti* can directly use dissolved oxygen to grow and to produce acetic acid, and, simultaneously, the environment becomes anaerobic or microaerobic, allowing *S. cerevisiae* to produce alcohol, which is then used by *A. aceti* as a substrate for the production of acetic acid.

According to Zhou et al. [29], agitation can cause shear forces that can influence changes in cell morphology, variations in the growth and formation of products, and damages to the cell structure. However, increasing the speed of agitation results in stronger mixing processes, more rapid contacts between nutrients and microbes,

and higher oxygen transfer rates (OTR) and oxygen uptake rates (OUR); therefore, aerobic and anaerobic environmental conditions are created simultaneously over a short period of time. Therefore, increasing agitation speed, up to a certain level, can lead to the production of larger amounts of acetic acid over shorter periods of time. In addition, the high dissolved oxygen content caused by the increased agitation speed in this system does not appear to cause oxidative stress or damage to proteins in cells, which could inhibit *A. aceti* growth [33]. As a whole, under conditions using an optimal inoculum ratio and an optimal agitation speed, the conversion efficiency from sugar to acetic acid increased to 362%.

3.4 The dynamics of changes in the sugar, alcohol, and acetic acid contents and in the pH value during fermentation under optimal conditions

The dynamics of changes in the sugar, alcohol, and acetic acid contents and in the pH values during the fermentation of acetic acid from apple juice under optimal conditions can be observed in **Figure 6**. In the beginning, when the sugar level is high, *S. cerevisiae* works to produce alcohol, increasing the alcohol contents. In conjunction with the production of alcohol, *A. aceti* began to produce acetic acid, causing the acetic acid level to increase. As *S. cerevisiae* produces alcohol, *A. aceti* simultaneously grows until the alcohol contents produced by *S. cerevisiae* are sufficient for *A. aceti* to produce acetic acid. In the mixed culture fermentation, *A. aceti* which is an obligate aerobic microbe uses dissolved oxygen for growth and for the oxidation of alcohol into acetic acid. However, the medium also undergoes an anaerobic state due to a lack of oxygen, allowing *S. cerevisiae* to convert sugar into alcohol. Another advantage of the use of mixed cultures is that the continuous consumption of oxygen by *A. aceti* appears to cause *S. cerevisiae* to grow without the multiplication of cell mass. Thus, the sugar present in the substrate can maximally be converted into alcohol by *S. cerevisiae*, and the alcohol can subsequently maximally be converted into acetic acid by *A. aceti*. However, at the end of the process, a decrease in the resulting acetic acid levels was observed. This decrease may be due to the unfavorable pH of the medium, which could inhibit the microbes from metabolizing substrates and producing acetic acid, or may be due to acetic acid overoxidation due to the limited

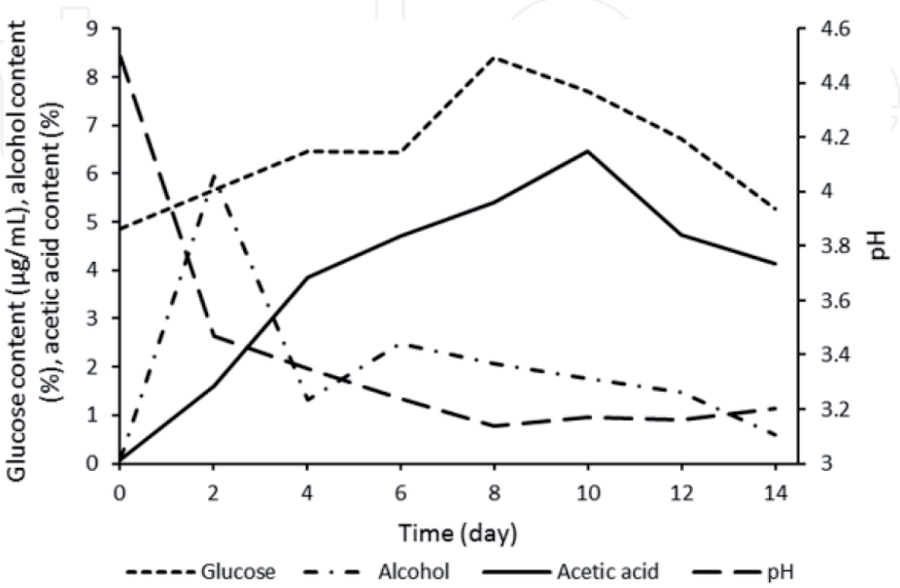


Figure 6.
The dynamics of the changes in glucose, alcohol, and acetic acid contents and pH values under optimal fermentation conditions.

availability of nutrients. For the purposes of harvesting the products, the fermentation process can be stopped when the highest yield is achieved.

3.5 Future outlook

Under the right conditions, the production of acetic acid can be maximized by using a simple system, such as submerged batch fermentation using a mixed culture that acts synchronously. Optimization can be performed by considering the character and needs of all microbes involved, which are the nutritional adequacy of the medium, the microbial proportions in the inoculum, and the agitation speed. The use of a mixed culture could shorten the fermentation time, reduce fermentation losses, and increase the acetic acid yields [16]. Some other advantages of this system compared with a gradual system are the relatively simple operation and easy handling of this system, which no particular control is required during the fermentation process, and the low risk of contamination. Thus, the application of this system for industrial purposes can be considered. However, the future scaling up of this process should consider other factors, including automation systems and the use of cutting-edge technologies in both the production and monitoring processes, to further improve the productivity and product quality without increasing production costs.

4. Conclusion

The efficiency of the acetic acid fermentation process can be assessed using a simplified system with mixed cultures. Some of the aspects evaluated in this system were the availability of sugar in the medium, the inoculum ratio of the cultures used, and the speed of agitation. By optimizing this system, the resulting acetic acid levels can be increased.

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